Comparative Biochemistry and Physiology, Part A 227 (2019) 116–122

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

DNA damage as an indicator of chronic stress: Correlations with corticosterone and uric acid

Brenna M.G. Gormally*, Rory Fuller, Mitch McVey, L. Michael Romero

Department of Biology, Tufts University, Medford 02155, MA, USA

ARTICLE INFO

Keywords:
DNA damage
Comet assay
Corticosterone
Uric acid
Captivity
Erythrocytes

ABSTRACT

Corticosterone does not change in consistent ways across species and contexts, making it challenging to use as an indicator of chronic stress. We assessed DNA damage as a potential metric that could be a more integrative stress measurement with direct links to health. We captured free-living house sparrows, took an immediate blood sample, and transferred them to the laboratory, exposing them to the chronic stress of captivity. Biweekly blood and weight samples were then taken for 4 weeks. We immediately assessed DNA damage in red blood cells using the comet assay and later quantified corticosterone. Uric acid was analyzed in a separate group of birds. We found that birds initially lost, but began to regain weight over the course of captivity. DNA damage peaked within the first 10 days of captivity, and mostly remained elevated. However, the cellular distribution of damage changed considerably over time; most cells showed low levels of damage early, a bimodal distribution of high and low DNA damage during the peak of damage, and a wide unimodal distribution of damage at the end of the 4 weeks. Furthermore, corticosterone increased and remained elevated and uric acid decreased and remained depleted over the same period. Although both a molecular (DNA damage) and an endocrine (corticosterone) marker showed similar response profiles over the 4 weeks, they were not correlated, suggesting they reflect different aspects of the underlying physiology. These data provide convincing preliminary evidence that DNA damage has potential to be an additional indicator of chronic stress.

1. Introduction

In recent decades, corticosterone (Cort) has become a focal point of many experiments aiming to assess stress in individual organisms and populations (e.g. Costantini et al., 2008; Deviche et al., 2014; Liebl and Martin, 2012; Love et al., 2017). Cort is a steroid hormone—a glucocorticoid—that is released from the adrenal glands, triggered by a cascade of signals from the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al., 2000). At baseline levels, it is responsible for a number of physiological and behavioral effects (see Landys et al., 2006 for a review). Despite these critical roles Cort plays at baseline levels, it is often most associated with its role as a 'stress hormone' as it becomes elevated within minutes of a vertebrate interacting with a stressful stimulus. For this reason, it has come to be used as the catch-all hormone for assessing the physiological state of an organism to determine how 'stressed' it is. Unfortunately, however, measuring circulating Cort in the blood only captures a portion of its function. Not only is a single blood sample a "snap shot" of a dynamic response, but receptors and binding globulins are also important for understanding Cort's impact. Examining a downstream consequence of Cort, thereby integrating multiple features of the Cort response, could provide a more logical, meaningful, and possibly consistent measurement.

Studying 'chronic stress' has also become of particular interest in a number of fields. For the purposes of this study, we are defining chronic stress as occurring when the physiology of an animal is strained past its 'normal range' (e.g. entering homeostatic overload; Romero et al., 2009) due to increased intensity or persistence of a disturbance. Many studies have attempted to use Cort as a bioindicator of underlying physiological condition, despite inconsistent trends. Some studies have found that Cort increases during chronic stress (e.g. Clinchy et al., 2004; Fischer et al., 2018; Lattin et al., 2012), while others have reported decreases (e.g. Cyr et al., 2007; Dickens et al., 2009) and still others have shown no differences (e.g. Angelier et al., 2016). In fact, a recent review of the literature found that Cort does not change in a consistent manner due to chronic stress in free-living animals (Dickens and Romero, 2013). Furthermore, it is likely that changes in Cort are context-specific, meaning that the type of chronic stress an animal experiences might alter the function and regulation of the HPA axis differently.

Although it is generally accepted that chronic stress—no matter its

* Corresponding author at: 200 College Avenue, Medford 02155, MA, United States.
E-mail address: brenna.gormally@tufts.edu (B.M.G. Gormally).

https://doi.org/10.1016/j.cbpa.2018.10.007
Received 5 October 2018; Accepted 5 October 2018
Available online 15 October 2018
1095-6433/

origin—causes broad physiological and behavioral consequences including immunosuppression (Dhabhar and McEwen, 1997; Martin, 2009), reduced reproductive success (Cyr and Romero, 2007; Dickens and Bentley, 2014), and altered behavior (Gormally et al., 2018; Love et al., 2016), it remains unclear whether Cort is the proximal mechanism for these responses. The broader field of stress physiology is in need of a more integrative and consistent biomarker of chronic stress (Romero et al., 2015). In this study, we present data that suggest one molecular technique could address this need. Cort is primarily a transcription regulator; circulating molecules bind to nuclear receptors that are present in nearly every tissue (Lattin et al., 2015), eliciting up or down regulation of a variety of genes. Because of Cort's close association with transcription and the maintenance of DNA, it is logical to explore more molecular biomarkers of chronic stress.

For this reason, we decided to investigate the effect of the introduction to captivity—a strong and persistent stressor—on DNA damage. Captivity has been shown to affect both physiology and behavior in animals (Fischer et al., 2018; Lattin et al., 2017, 2012) as it introduces a number of novel stimuli that they must cope with (Morgan and Tromborg, 2007). Furthermore, captivity has been shown to affect not just Cort, but also the sympathetic nervous system (Fischer et al., 2018), reproduction (Dickens and Bentley, 2014), immune function (Love et al., 2017), and behavior (Lattin et al., 2017), making it a compelling model to use when studying chronic stress. DNA damage has the potential to be a more integrative measurement of chronic stress because it occurs downstream of hormone concentrations. Damage could also have a direct impact on the underlying physiology of an individual by altering the ability to transcribe vital genes. Prior studies have already drawn connections between DNA damage and stress-related hormones, both during the acute and chronic responses. DNA damage has been shown to become significantly elevated within 10 min of exposure to Cort, epinephrine, and norepinephrine in vitro (Flint et al., 2007). This same study showed that concurrent addition of blockers of these hormones results in no elevations in damage, suggesting a direct link between them and genomic damage. While this study used acute (short-term) exposures to stress-related hormones, the results still suggest value for using DNA damage in chronic situations. Following our working definition, chronic stress can result from either the duration or intensity of acute stressors; in other words experiencing layered acute stressors can be similarly detrimental to the physiology of an animal as a persistent chronic stressors (e.g. Cyr et al., 2007; Cyr and Romero, 2007; Gormally et al., 2018) A separate study showed that when mice are chronically infused with isoproterenol, a β-2-adrenoreceptor agonist, there is a marked increase in DNA damage and genome instability (Hara et al., 2011). These two studies suggest that DNA damage is linked to both the Cort and epinephrine/norepinephrine arms of the stress response, making it an attractive avenue to explore.

Though DNA damage has been predominantly assessed in lab-based rodent models, there have been a number of studies of wild animals that have measured damage as part of toxicology studies in the field (Bonisoli-Alquati, 2014). This has become a valuable tool to use to assess the impacts of toxic disasters like radiation, oil, and chemical spills. In general, studies have detected significantly elevated DNA damage levels in birds in regions afflicted by these kinds of disasters (e.g. Baos et al., 2006; Maness and Emslie, 2001; Pastor et al., 2004; Sicolo et al., 2010). Some other studies have shown that different types of stress (chronic and acute) can impact DNA damage in fish, reptilian, and avian species (Malandrakis et al., 2016; Meitern et al., 2013; Schwartz and Bronikowski, 2013). Despite the number of studies that have assessed DNA damage in vertebrates, proper validations have not been conducted for correlating it to stress; instead, it has been assumed that higher DNA damage is associated with more "stress," similar to the associations drawn with Cort.

To more directly assess whether DNA damage could be an indicator of chronic stress, we brought house sparrows (*Passer domesticus*) into captivity and measured Cort, DNA damage, and weight twice a week for 4 weeks. Because avian erythrocytes are nucleated and have a shorter lifespan than their mammalian counterparts (Beuchat and Chong, 1998), we could easily and repeatedly sample the same individual over the course of captivity to assess DNA damage. In a separate group of birds, uric acid was also assessed throughout the introduction to captivity. Uric acid is a potent antioxidant (Ames et al., 1981) and thus changes could reflect alterations to the redox balance during captivity (Cohen et al., 2007). Oxidative stress is common in erythrocytes so measuring uric acid could provide one lens through which to examine the aspect of damage (Mohanty et al., 2014; Sinha et al., 2015). Uric acid also has direct links to Cort, being shown to increase following acute and chronic exposures (Lin et al., 2004a, 2004b). We have previously shown, however, that uric acid decreases during periods of chronic stress (separate from the administration of exogenous Cort) (Gormally et al., 2018). We predicted that baseline Cort and erythrocyte DNA damage would increase, and uric acid decrease, upon introduction to captivity; and that all three metrics would stabilize once the birds acclimated to captivity. Consequently, this study fills the critical gap of determining if the magnitude of DNA damage is correlated with the duration or magnitude of stress; prior studies only support the relationship between Cort administration and DNA damage.

2. Materials and methods

2.1. Experimental design

Two groups of house sparrows, Group 1 of 9 birds (4 females and 5 males; April 2018) and Group 2 of 10 birds (5 females and 5 males; October 2017), were passively captured by mist nets and potter traps in Medford, MA, USA. Within 3 min of capture, a blood sample (~45 uL) was taken from the alar vein and collected in a heparinized capillary tube. Samples collected within this 3-min window have been shown to reflect baseline Cort concentrations (Romero and Reed, 2005). Birds were then brought into captivity, where they were housed individually in cages (45 cm × 37 cm × 33 cm) on a 12 L:12D light cycle.

Blood samples were taken in the same fashion as the pre-captivity sample on days 3, 6, 10, 13, 17, 20, 24, and 27 of captivity. Birds were also weighed on each of these days at the time of sampling. Blood samples were kept on ice until processing, within 2 h of collection. For Group 1, a portion of whole blood was diluted in phosphate buffered saline (Ca^{2+}, Mg^{2+} free) for use in the comet assay prior to centrifugation (see below). For both groups, the remaining, undiluted blood samples were then separated by centrifugation. Aliquots of plasma were frozen at −20 °C for later Cort quantification (Group 1) or uric acid (Group 2).

This experiment was approved by the Tufts Institutional Animal Care and Use Committee and was conducted in compliance with the Guidelines for Use of Wild Birds in Research (Fair et al., 2010).

2.2. Comet assays

DNA damage was assessed in the erythrocytes from the whole blood samples of Group 1 house sparrows using a comet assay performed within 5 h of blood collection. Low melt agarose (LMA), lysis solution, control cells, and CometSlides were purchased from Trevigen (Gaithesburg, MD, USA). Before starting the assay, the LMA was melted in a beaker of boiling water; it was then cooled in a 37 °C water bath for 20 min. At the same time, the appropriate number of CometSlides (20-well) were warmed in a 37 °C drying oven. To avoid the accumulation of dust, slides were kept in closed petri dishes when in the oven. Once cooled, 300 μL LMA was added to pre-warmed microcentrifuge tubes (1.5 mL) tubes—one for each sample. These tubes were kept in a heated dry block to keep the LMA melted.

All of the following steps were conducted in dim light or in the dark to avoid excess UV-induced damage to the erythrocytes. Whole blood (collected and separated prior to centrifugation) was diluted 400-fold in

phosphate buffered saline (1×, pH 7.4, Ca^{2+}, Mg^{2+} free; e.g. 2 μL plasma into 800 μL PBS). Two additional 5-fold dilutions were then created for each sample. This large dilution is necessary to get a reasonable number of cells to quantify damage. Too low a dilution will result in far too many cells being on the CometSlide that will overlap and make imaging and analysis impossible. For each sample, 30 μL of this final dilution was added to the pre-warmed 300 μL LMA. After quickly mixing, 30 μL was added to the CometSlide in duplicate. The sample was then completely spread onto the well using the side of the pipette tip. This process was repeated for each sample.

The CometSlide was then placed in a 4 °C refrigerator in the dark for 30 min to solidify the gel. After this period, the slide was covered in lysis buffer (40 mL Trevigen buffer, 4 mL DMSO) and left in the refrigerator for 1 h. The slide was then removed from the buffer and submerged in electrophoresis buffer (500 mM sodium acetate, 100 mM Tris base, pH 10) for 30 min at 6 °C. Note that this is a less basic pH than the more common alkaline comet assay. The alkaline comet assay detects both single and double stranded DNA breaks while this less basic versions only detect double stranded breaks. Upon initial trials of the alkaline comet assay (pH > 13), we found that nearly all DNA migrated into the tail of the comet, making it impossible to analyze differences. It has been qualitatively reported that avian erythrocytes contain high numbers of alkali labile sites, which are then converted to strand breaks during alkaline treatment (Bonisoli-Alquati et al., 2010; Galván et al., 2014). The neutral version of the assay greatly reduced the frequency of these overly-damaged comets, since it likely does not convert these alkali labile sites and detects a higher proportion of double strand breaks (see reviews Collins, 2004; Collins et al., 2008; Langie et al., 2015). Electrophoresis was then performed at 21 V for 30 min at 6 °C (using 850 mL of buffer). Two 5-min washes with chilled distilled water were then conducted followed by a 5-min wash with chilled 70% ethanol. Finally, the slide was dried in a covered petri dish in a 37 °C drying oven. The slide was considered to be completely dried once the gel disappeared.

Slides were stored in a cool, dark box with desiccant. When ready to image, slides were removed from the box and stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) for 30 min. Excess stain was removed with distilled water and slides were once again dried. Slides were then imaged using the 10× objective on a fluorescent microscope.

Four control samples were run during each assay as well. These control samples were purchased from Trevigen (Neutral Control Cells) and treated in the same way as the samples. The control cells have increasing amounts of pre-set damage and therefore allow for consistency between assays; the inter-assay CV based on these samples was 22.6%.

2.3. Corticosterone radioimmunoassays

Cort concentrations were measured from Group 1 birds using a radioimmunoassay (Wingfield et al., 1992). In brief, ~20 μL of plasma was diluted to 200 μL with distilled water. Each sample was then spiked with 20 μL of tritiated Cort to assess steroid extraction efficacy. Steroids were extracted with 4 mL of dichloromethane. These extracts were dried using nitrogen and then rehydrated with phosphate buffered saline with added gelatin. The radioimmunoassay was then conducted with the B3–163 antibody (Esoterix, Calabasas Hills, CA, USA) and tritiated Cort. Assay sensitivity was 1.07 ng/mL and the inter and intra-assay CVs were 9% and 3% respectively. Samples that fell below the limit of detection (5 of 78 samples) were assigned the floor of the assay (1.07 ng/mL).

2.4. Uric acid comparisons

Uric acid was analyzed from Group 2 blood samples using an Amplex® Red Uric Acid/Uricase Assay Kit (Molecular Probes, Eugene, OR, USA). Assays were conducted according to the manufacturer's instructions. Samples were run in triplicate and the inter and intra-assay CVs were 3.9% and 3.0% respectively.

2.5. Data analysis

All statistical analyses were conducted in RStudio (RStudio Team, 2015).

Changes in weight were assessed by calculating percent of initial, pre-captivity weights. These initial values were considered to be 100% and any decreases or increases in weight were reported as changes from 100%.

Comet assay images were analyzed using the OpenComet (Gyori et al., 2014) plugin for Fiji (Schindelin et al., 2012). All images were analyzed by the same individual (BG) who was blinded to the exact day of the experiment. This open-access program enables faster, less subjective analysis of comets. The program automatically identifies comets while also allowing for a user to manually exclude aberrant comets. It produces a spreadsheet with 16 different parameters for each individual comet. We extracted "TailDNA%," which is the percentage of DNA that has migrated into the tail of the comet. A higher TailDNA% thus correlates to increased DNA damage. We analyzed the average TailDNA% for each bird at each day of captivity, but also assessed the distribution of damage at each of these sampling points. To clarify, we assessed the distribution of damage of all the cells, from all the samples taken on a given day of captivity, generating 8 different distributions. The modality of these distributions was analyzed using Hartigan's dip test (diptest package, Maechler, 2016). There is currently not a consensus on the best way to assess bimodality, but Hartigan's dip test is a general test of multimodality. A p-value of < 0.05 suggests that the distribution is not unimodal, and at least bimodal (Hartigan and Hartigan, 1985).

The effect of captivity day on mass, DNA damage, Cort, and uric acid was assessed using linear mixed effects models ('lmer' function, lme4 package, Bates et al., 2015) with individual bird identity included as a random effect. The effect of sex was also checked using a separate model where sex was included as an interaction term. Cort concentrations were log transformed and TailDNA% was square transformed to maintain homogeneity of variances, which was tested using Levene's Test. We then used the 'Anova' function of the car package (Fox and Weisberg, 2011) to test for statistical significance of the linear model. In the event of a significant result, the 'glht' function of multcomp package was used to test for pairwise differences between the pre-captivity measurement and each day of captivity (Hothorn et al., 2008). Residual plots were visually inspected for each linear model. Finally, we assessed the correlation between Cort and DNA damage using a Pearson correlation.

3. Results

3.1. Weight

House sparrows lost a significant amount of weight by day 6 of captivity ($F_{8,\ 72} = 4.79$, $p < .0005$; Fig. 1). Weight remained significantly reduced compared to pre-captivity levels through day 27 of captivity. Females and males lost weight in a similar fashion (main effect, $F_{1,\ 79} = 0.00$, $p = 1.00$; interaction, $F_{8,72} = 0.87$, $p = .55$)

3.2. DNA damage

DNA damage varied over the course of captivity, increasing from pre-captivity levels ($F_{8,\ 72} = 29.08$, $p < .0001$; Fig. 2). DNA damage peaked at day 10 of captivity (45.90 ± 3.01%). Following day 10, DNA damage initially decreased to pre-captivity levels on day 20, but then increased again on days 24 and 27. Changes in DNA damage was not influenced by sex (main effect, $F_{1,79} = 0.38$, $p = .54$; interaction, $F_{8,72} = 0.23$, $p = .64$)

The shape of the distributions of the DNA damage also shifted over

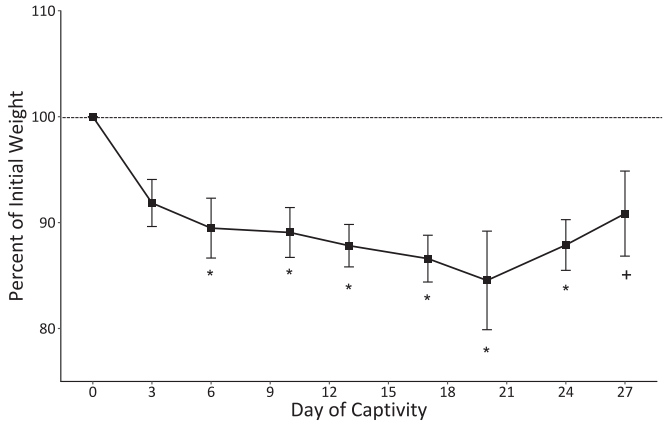

Fig. 1. Change in weight of house sparrows over the course of captivity. Changes were calculated as the percent change from initial, pre-captivity weights, with 100% assigned as the pre-captivity weight. + indicates significance at $p < .05$ while * indicates significance at $p < .01$. Error bars represent ± SEM from $n = 9$ birds.

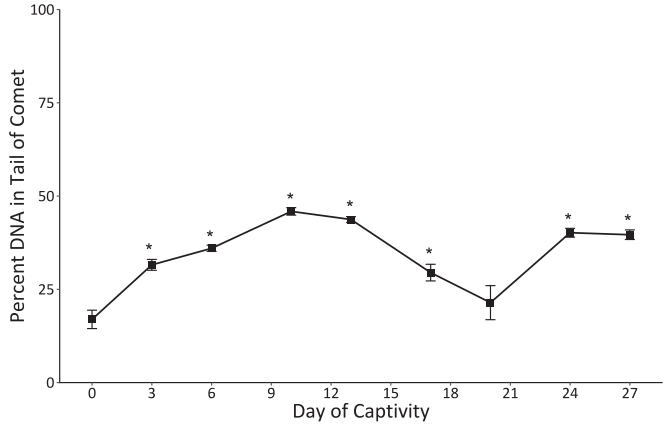

Fig. 2. DNA damage in the erythrocytes of house sparrows changes due to captivity. * indicates significance at p < .01. Error bars represent ± SEM from n = 9 birds.

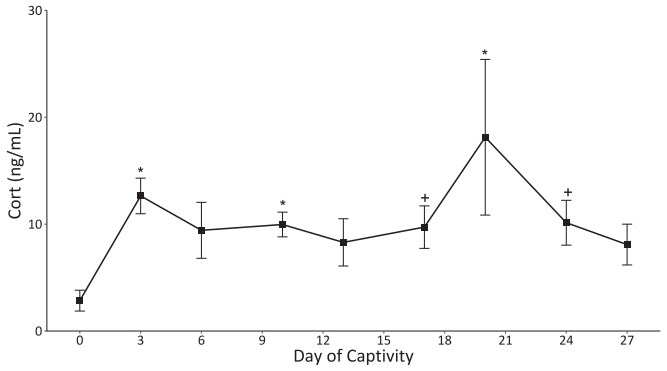

Fig. 4. Corticosterone in house sparrows brought into captivity. + indicates significance at p < .05 while * indicates significance at p < .01. Error bars represent ± SEM for n = 9 birds.

the course of the captivity (Fig. 3). These distributions were assessed by combining for data of all the cells, from all individuals at each day of captivity. At capture, DNA damage was visually unimodally distributed. A bimodal—or at least non-unimodal—distribution then emerged at days 10 and 13. Results from Hartigan's dip test suggests non-unimodal distributions on days 10 and 13 (Fig. 3).

3.3. Corticosterone

Pre-captivity baseline Cort concentrations were often so low they were undetectable, but baseline Cort increased dramatically upon transference to captivity within 3 days and remained high, never returning to pre-captivity levels ($F_{8,\ 69} = 3.73$, $p < .005$; Fig. 4). Cort was not significantly correlated to DNA damage ($\rho = 0.16$, $p = .17$) and females and males experienced similar Cort changes (main effect, $F_{1,76} = 0.8$, $p = .37$; interaction, $F_{8,69} = 0.38$, $p = .93$).

3.4. Uric acid

Uric acid decreased significantly within the first 3 days of captivity ($F_{10,\ 85} = 11.01$, $p < .0001$; Fig. 5). Plasma uric acid remained depleted throughout the entire 4 weeks of captivity and there were no

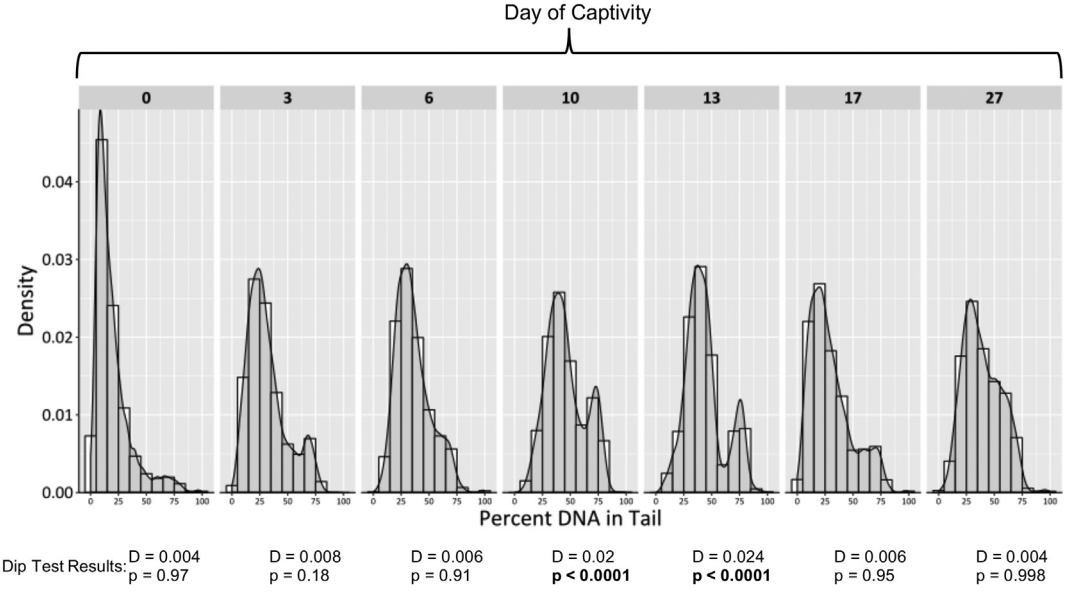

Fig. 3. Density plots of the percent of DNA in the tail of the comets upon introduction to captivity. Plot includes histograms of all 9 birds at each time point with overlaid kernel density curves. Dip test results are shown below the x-axis. Bolded numbers indicate values that are below a significance value of 0.05. Day 24 has been omitted for space purposes.

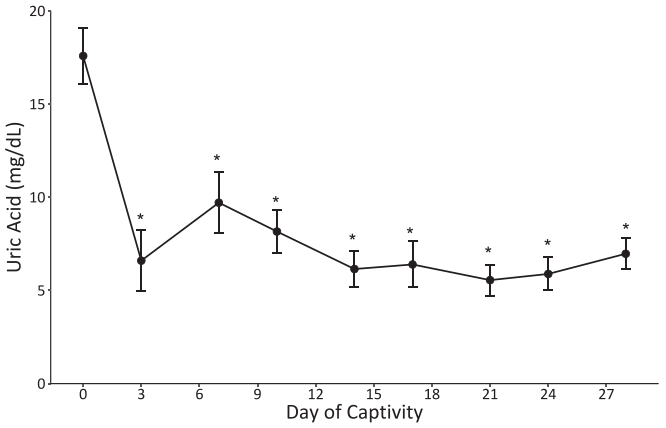

Fig. 5. Uric acid changes in response to captivity. * indicates significance at $p < .01$. Error bars represent ± SEM for $n = 10$ birds.

differences in females and males (main effect, $F_{1,77} = 0.03$, $p = .88$), however there was a weakly significant interaction between sex and day of captivity ($F_{8,70} = 2.26$, $p = .04$) driven by females who experienced more rapid reductions in uric acid.

4. Discussion

In this study, we tested how DNA damage measured in avian erythrocytes changes in response to a known persistent chronic stressor—captivity. DNA damage increases and remains variably high over the course of captivity. Another physiological parameter, body weight, shows a similar pattern, with birds initially losing weight, but beginning to regain that weight towards the end of the experiment. Interestingly, Cort abruptly changed when the house sparrows were brought into captivity, but levelled off and did not substantially change after the first week of captivity. Finally, uric acid dropped dramatically and remained depleted over the same time course. These results highlight that different physiological mediators adjust to captivity over different timescales. Furthermore, they indicate that DNA damage can be a valuable addition to assess chronic stress in conjunction with other physiological.

4.1. Average DNA damage changes as a result of captivity

We found that house sparrows brought into captivity experienced elevated DNA damage within 3 days (Fig. 2). Two lines of evidence suggest that this damage is likely the result of oxidative stress. First, captivity creates a challenging environment that organisms must adapt to (Morgan and Tromborg, 2007), and coping with challenges results in increased energy expenditure (Cyr et al., 2009). Oxidative stress occurs when too many free radicals are produced and the body's antioxidant defenses are unable to counteract their effects; these free radicals can, in turn, lead to damaged DNA (Bayir, 2005). The majority of free radicals are produced as byproducts of the electron transport chain, so that an increase in cellular metabolism will have a concomitant increase in free radical production (Cohen et al., 2008; Huang and Manton, 2004). Though there has not been extensive research into the metabolic functions of avian erythrocytes, they do produce reactive oxygen species which could lead directly to DNA damage (Zhang et al., 2011). Red blood cells are also known to be exposed to reactive oxygen species in circulation (not necessarily those produced by themselves) and these can lead to the accumulation of damage and eventual senescence (Mohanty et al., 2014; Sinha et al., 2015).

The second line of evidence that DNA damage results from oxidative stress comes from the concomitant increase in circulating baseline Cort (Fig. 4) that matches previous studies in house sparrows transferred to captivity (Fischer et al., 2018; Kuhlman and Martin, 2010; Love et al., 2016). In these studies, Cort remained elevated to different degrees, but typically never returned to pre-captivity levels. Cort treatment on both acute (Lin et al., 2004a) and chronic timescales (Costantini et al., 2011, 2008; Lin et al., 2004b) alter levels of antioxidants, leading to oxidative stress. Consequently, elevations in Cort likely lead directly to oxidative stress and subsequent DNA damage. Note that while Cort and DNA damage change in visually similar ways (Figs. 2 and 3), we found they were not directly correlated, suggesting they capture independent mechanisms.

The potential connection to oxidative stress led us to subsequently partially test this hypothesis by measuring uric acid, a potent antioxidant in avian species. The results from the separate group of birds shows that uric acid rapidly decreases upon introduction to captivity (Fig. 5). Other studies using a variety of stress protocols have shown that chronic stress can lead to alterations in uric acid (Cohen et al., 2008; Gormally et al., 2018). In addition to its antioxidant roles, uric acid is also the end product of purine metabolism in birds (Cohen et al., 2007). Changes in uric acid —and other antioxidants—during periods of chronic stress possibly represent depletion of antioxidant stores as a means to partially cope with the ongoing oxidative stress. In the absence of chronic stress, uric acid levels tend to be quite stable in house sparrows (Pap et al., 2015).

Our working model is that captivity results in increased oxidative stress resulting from increases in circulating reactive oxygen species, that in turn leads to initial increases in DNA damage. This process appears to peak at about 10 days. By 2.5–3 weeks, however, the birds have essentially acclimated to their new captive environment. Prior studies on house sparrows introduced to captivity suggested that both Cort and heart rate (a proxy for epinephrine/norepinephrine) reach new plateaus within a similar time frame (Fischer et al., 2018). The results of this study also show that both Cort and uric acid level off after a relatively brief period of time. Once these new baselines have been reached, we hypothesize that there is a reduction in the number of reactive oxygen species in circulation. The older erythrocytes that became damaged during those initial few weeks are removed from circulation by erythrophagocytosis and replaced with younger, relatively undamaged cells (Arias and Arias, 2017). Even though Cort remains elevated and uric acid remains depleted, the DNA damage results suggest that acclimation may have begun within 3 weeks (see Day 20, Fig. 2). In support of this hypothesis, weight begins to trend back to initial levels at approximately the same time, suggesting that birds may have begun to acclimate. Interestingly, DNA damage began to increase once again by 3.5–4 weeks in captivity. We do not yet have a hypothesis for these complex temporal dynamics, however no other physiological parameters changed during this time period, suggesting that the birds were not reentering a new period of chronic stress, but rather that new baselines had been established.

Though avian erythrocytes are terminally differentiated, they do in fact have functional mitochondria (Stier et al., 2013) and have even been shown to be capable of transcription (Morera et al., 2011). Therefore, the damage they incur as a result of oxidative stress or other mechanisms is likely meaningful. Though not yet explored, erythrocyte damage could also be reflective of damage in other tissues that might be more detrimental to overall health.

4.2. Changes in the distribution of DNA damage in comets

In addition to the changes we observed in the average DNA damage over the course of the experiment, we also found unique shifts in the distributions of damage within the individual samples. We noticed that a visual shift in damage distribution occurs in the histograms of damage at later sampling points. When birds are initially sampled in the wild, the distribution of the damage in their erythrocytes is visually normal, and any skew is biased towards lower damage. In fact, the majority of comets have < 25% of DNA in their tails. However, over the course of the first few weeks in captivity, this unimodal distribution shifts to a

more bimodal distribution (Fig. 3). These data suggest that there could be two different population of red blood cells in circulation at the start of the experiment—older erythrocytes that are more susceptible to damage and younger cells that are less susceptible. Upon introduction to captivity, the more susceptible cells become damaged more quickly than the less susceptible cells. The younger cells still accumulate some DNA damage, however to a lesser degree. Thus a bimodal distribution of heavily damaged (more susceptible) and lesser damaged (less susceptible) cells emerges after the initial 1.5–2 weeks in captivity. These data suggest that older erythrocytes with more DNA damage are being replaced by younger, less damaged ones over time, leaving a population of heavily damaged cells (older) and those that are only moderately damaged (newer). Eventually, this bimodal pattern returns to a roughly unimodal distribution (Days 17 and 28, Fig. 3), although with a much broader range indicating substantial numbers of damaged cells remain. At this point, the entire population of red blood cells consists of a mixture of brand new (as of introduction to captivity) and older cells that have been damaged and aged throughout captivity. Note that even after 27 days, the distribution is quite different than the distribution at capture, with most comets having > 25% DNA in their tails. The change in distribution, however, suggests a way to visually observe the acclimation process. Avian red blood cells naturally have a life span of about 35–45 days (Beuchat and Chong, 1998), however erythrocyte life span is flexible and can be tuned to the amount of oxygen in the environment (Arias and Arias, 2017). This shift in distribution shape could also therefore be reflecting the natural aging process of erythrocytes.

4.3. Implications for future stress physiology studies

Perhaps the most interesting of these results is that DNA damage is peaking within a 10-day window (Fig. 2). Prior studies involving human-induced chronic stress protocols indicate that significant changes in baseline and stress-induced Cort as well as strength of HPA axis negative feedback begin to occur after 10–12 days (Cyr et al., 2007; Cyr and Romero, 2007; Lattin and Romero, 2014; Rich and Romero, 2005). While it's important to note that these chronic stress protocols and introduction to captivity are different types of chronic stress, it is promising that DNA damage points to a similar time frame. We believe these data support the idea that animals enter homeostatic overload (Romero et al., 2009) within this time frame, but that acclimation likely begins to occur following this in captivity as no additional stressors are being experienced.

Measures of DNA damage should be considered in other studies aiming to assess chronic stress in individual organisms, particularly in avian species. We would like to emphasize that while DNA damage and Cort did show similar profiles in this study, they measure distinct processes; in the future, DNA damage may prove to be a more consistent measurement as it circumvents many of the difficulties of quantifying Cort (e.g. receptor and binding globulin dynamics). Measuring DNA damage with the comet assay is simple, allows for repeated measures designs, and economical. It could be measured in conjunction with other physiological parameters relatively easily. It has become increasingly obvious that Cort plays a complicated role that often isn't consistent, particularly during periods of chronic stress. Therefore, measuring this hormone alone is not sufficient to understanding the complex changes that chronic stress induces. These data suggest that DNA damage is a useful metric to assess alongside other physiological markers. It is becoming increasingly clear that these markers change over different timescales and that the type of chronic stress heavily influences these changes (Fischer et al., 2018; Gormally et al., 2018). We feel that a multi-modal approach should be used whenever possible, and advocate that DNA damage quantification should become part of our physiological toolkit.

Acknowledgements

We would like to thank the Tufts University Division of Laboratory Animal Medicine staff for their care of the animals involved in this study. We would also like to thank Sidney Beecy and Hannah Yin for their assistance in sampling, Jenna Whalen for her help with microscopy, and two anonymous reviewers for their valuable feedback. This study was funded by National Science Foundation grant IOS 1655269 to LMR and a Tufts University Graduate Student Research Award to BMGG.

References

Ames, B.N., Cathcart, R., Schwiers, E., Hochstein, P., 1981. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. Proc. Natl. Acad. Sci. U. S. A. 78, 6858–6862.

Angelier, F., Meillère, A., Grace, J.K., Trouvé, C., Brischoux, F., 2016. No evidence for an effect of traffic noise on the development of the corticosterone stress response in an urban exploiter. Gen. Comp. Endocrinol. 232, 43–50.

Arias, C.F., Arias, C.F., 2017. How do red blood cells know when to die? 4 R. Soc. Open Scihttps://doi.org/10.1098/rsos.160850.

Baos, R., Jovani, R., Pastor, N., Tella, J.L., Jiménez, B., Gómez, G., González, M.J., Hiraldo, F., 2006. Evaluation of genotoxic effects of heavy metals and arsenic in wild nestling white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) from Southwestern Spain after a mining accident. Environ. Toxicol. Chem. 25, 2794–2803. https://doi.org/10.1897/05-570R.1.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48.

Bayir, H., 2005. Reactive oxygen species. Crit. Care Med. 33, S498–S501. https://doi.org/10.1097/01.CCM.0000186787.64500.12.

Beuchat, C.A., Chong, C.R., 1998. Hyperglycemia in hummingbirds and its consequences for hemoglobin glycation. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 120, 409–416.

Bonisoli-Alquati, A., 2014. Avian genetic ecotoxicology: DNA of the canary in a coalmine. Curr. Zool. 60, 285–298.

Bonisoli-Alquati, A., Voris, A., Mousseau, T.A., Møller, A.P., Saino, N., Wyatt, M.D., 2010. DNA damage in barn swallows (*Hirundo rustica*) from the Chernobyl region detected by use of the comet assay. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 151, 271–277.

Clinchy, M., Zanette, L., Boonstra, R., Wingfield, J.C., Smith, J.N.M., 2004. Balancing food and predator pressure induces chronic stress in songbirds. Proc. R. Soc. Lond. B Biol. Sci. 271, 2473–2479.

Cohen, A., Klasing, K., Ricklefs, R., 2007. Measuring circulating antioxidants in wild birds. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 147, 110–121.

Cohen, A.A., Hau, M., Wikelski, M., 2008. Stress, metabolism, and antioxidants in two wild passerine bird species. Physiol. Biochem. Zool. 81, 463–472.

Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol. Biotechnol. 26, 249–261.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151. https://doi.org/10.1093/mutage/gem051.

Costantini, D., Fanfani, A., Dell'Omo, G., 2008. Effects of corticosteroids on oxidative damage and circulating carotenoids in captive adult kestrels (*Falco tinnunculus*). J. Comp. Physiol. B. 178, 829–835. https://doi.org/10.1007/s00360-008-0270-z.

Costantini, D., Marasco, V., Møller, A.P., 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B. 181, 447–456. https://doi.org/10.1007/s00360-011-0566-2.

Cyr, N.E., Romero, L.M., 2007. Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. Gen. Comp. Endocrinol. 151, 82–89.

Cyr, N.E., Earle, K., Tam, C., Romero, L.M., 2007. The effect of chronic psychological stress on corticosterone, plasma metabolites, and immune responsiveness in European starlings. Gen. Comp. Endocrinol. 154, 59–66.

Cyr, N.E., Dickens, M.J., Romero, L.M., 2009. Heart rate and heart-rate variability responses to acute and chronic stress in a wild-caught passerine bird. Physiol. Biochem. Zool. 82, 332–344.

Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S., Valle, S., 2014. Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. Gen. Comp. Endocrinol. 203, 120–131.

Dhabhar, F.S., McEwen, B.S., 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav. Immun. 11, 286–306.

Dickens, Delehanty, D.J., Romero, L.M., 2009. Stress and translocation: alterations in the stress physiology of translocated birds. Proc. R. Soc. Lond. B Biol. Sci. 276, 2051–2056.

Dickens, M.J., Bentley, G.E., 2014. Stress, captivity, and reproduction in a wild bird species. Horm. Behav. 66, 685–693.

Dickens, M.J., Romero, L.M., 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. Gen. Comp. Endocrinol. 191, 177–189.

Fair, J., Paul, E., Jones, J. (Eds.), 2010. Guidelines to the Use of Wild Birds in Research. Ornithological Council, Washington, D.C..

Fischer, C.P., Wright-Lichter, J., Romero, L.M., 2018. Chronic stress and the introduction to captivity: How wild house sparrows (*Passer domesticus*) adjust to laboratory conditions. Gen. Comp. Endocrinol. 259, 85–92. https://doi.org/10.1016/J.YGCEN.2017.11.007.

Flint, M.S., Baum, A., Chambers, W.H., Jenkins, F.J., 2007. Induction of DNA damage, alteration of DNA repair and transcriptional activation by stress hormones. Psychoneuroendocrinology 32, 470–479.

Fox, J., Weisberg, S., 2011. An {R} Companion to Applied Regression, Second. ed. Sage, Thousand Oaks, CA.

Galván, I., Bonisoli-Alquati, A., Jenkinson, S., Ghanem, G., Wakamatsu, K., Mousseau, T.A., Møller, A.P., 2014. Chronic exposure to low-dose radiation at Chernobyl favours adaptation to oxidative stress in birds. Funct. Ecol. 28, 1387–1403. https://doi.org/10.1111/1365-2435.12283.

Gormally, B.M.G., Wright-Lichter, J., Reed, J.M., Romero, L.M., 2018. Physiological and behavioral responses of house sparrows to repeated stressors. PeerJ 6, e4961. https://doi.org/10.7717/peerj.4961.

Gyori, B.M., Venkatachalam, G., Thiagarajan, P.S., Hsu, D., Clement, M.-V., 2014. OpenComet: an automated tool for comet assay image analysis. Redox Biol. 2, 457–465. https://doi.org/10.1016/J.REDOX.2013.12.020.

Hara, M.R., Kovacs, J.J., Whalen, E.J., Rajagopal, S., Strachan, R.T., Grant, W., Towers, A.J., Williams, B., Lam, C.M., Xiao, K., Shenoy, S.K., Gregory, S.G., Ahn, S., Duckett, D.R., Lefkowitz, R.J., 2011. A stress response pathway regulates DNA damage through β2-adrenoreceptors and β-arrestin-1. Nature 477, 349–353.

Hartigan, J.A., Hartigan, P.M., 1985. The dip test of unimodality. Ann. Stat. 13, 70–84. https://doi.org/10.1214/aos/1176346577.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363.

Huang, H., Manton, K.G., 2004. The role of oxidative damage in mitochondia during aging: a review. Front. Biosci. 1100–1117. https://doi.org/10.2741/1298.

Kuhlman, J.R., Martin, L.B., 2010. Captivity affects immune redistribution to skin in a wild bird. Funct. Ecol. 24, 830–837. https://doi.org/10.1111/j.1365-2435.2010.01710.x.

Landys, M.M., Ramenofsky, M., Wingfield, J.C., 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. Gen. Comp. Endocrinol. 148, 132–149. https://doi.org/10.1016/J.YGCEN.2006.02.013.

Langie, S.A.S., Azqueta, A., Collins, A.R., 2015. The comet assay: past, present, and future. Front. Genet. 6, 266.

Lattin, C.R., Romero, L.M., 2014. Chronic stress alters concentrations of corticosterone receptors in a tissue-specific manner in wild house sparrows (*Passer domesticus*). J. Exp. Biol. 217, 2601–2608.

Lattin, C.R., Bauer, C.M., de Bruijn, R., Romero, L.M., 2012. Hypothalamus–pituitary–adrenal axis activity and the subsequent response to chronic stress differ depending upon life history stage. Gen. Comp. Endocrinol. 178, 494–501.

Lattin, C.R., Keniston, D.E., Reed, J.M., Romero, L.M., 2015. Are receptor concentrations correlated across tissues within individuals? A case study examining glucocortiocid and mineralocorticoid receptor binding. Endocrinology 156, 1354–1361.

Lattin, C.R., Pechenenko, A.V., Carson, R.E., 2017. Experimentally reducing corticosterone mitigates rapid captivity effects on behavior, but not body composition, in a wild bird. Horm. Behav. 89, 121–129.

Liebl, A.L., Martin, L.B., 2012. Exploratory behaviour and stressor hyper-responsiveness facilitate range expansion of an introduced songbird. Proc. R. Soc. Lond. B Biol. Sci. 279.

Lin, H., Decuypere, E., Buyse, J., 2004a. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) 2. Short-term effect. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 139, 745–751.

Lin, H., Decuypere, E., Buyse, J., 2004b. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) 1. Chronic exposure. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 139, 737–744.

Love, A.C., Foltz, S.L., Adelman, J.S., Moore, I.T., Hawley, D.M., 2016. Changes in corticosterone concentrations and behavior during *Mycoplasma gallisepticum* infection in house finches (*Haemorhous mexicanus*). Gen. Comp. Endocrinol. 235, 70–77.

Love, A.C., Lovern, M.B., DuRant, S.E., 2017. Captivity influences immune responses, stress endocrinology, and organ size in house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 252, 18–26.

Maechler, M., 2016. Diptest: Hartigan's Dip Test Statistic for Unimodality - Corrected.

Malandrakis, E.E., Dadali, O., Golomazou, E., Kavouras, M., Dailianis, S., Chadio, S., Exadactylos, A., Panagiotaki, P., 2016. DNA damage and differential gene expression associated with physical stress in gilthead seabream (*Sparus aurata*). Gen. Comp. Endocrinol. 236, 98–104. https://doi.org/10.1016/j.ygcen.2016.07.009.

Maness, T.J., Emslie, S.D., 2001. An analysis of possible genotoxic exposure in adult and juvenile royal terns in North carolina. Waterbirds Int. J. Waterbird Biol. 24, 352–360.

Martin, L.B., 2009. Stress and immunity in wild vertebrates: Timing is everything. Gen. Comp. Endocrinol. 163, 70–76.

Meitern, R., Sild, E., Lind, M.-A., Männiste, M., Sepp, T., Karu, U., Hõrak, P., 2013. Effects of endotoxin and psychological stress on redox physiology, immunity and feather corticosterone in greenfinches. PLoS One 8, e67545.

Mohanty, J.G., Nagababu, E., Rifkind, J.M., 2014. Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. Front. Physiol. 5, 1–6. https://doi.org/10.3389/fphys.2014.00084.

Morera, D., Roher, N., Ribas, L., Balasch, J.C., Doñate, C., Callol, A., Boltaña, S., Roberts, S., Goetz, G., Goetz, F.W., MacKenzie, S.A., 2011. RNA-Seq Reveals an integrated immune response in nucleated erythrocytes. PLoS One 6. https://doi.org/10.1371/journal.pone.0026998.

Morgan, K.N., Tromborg, C.T., 2007. Sources of stress in captivity. Appl. Anim. Behav. Sci. 102, 262–302.

Pap, P.L., Pătraş, L., Osváth, G., Buehler, D.M., Versteegh, M.A., Sesarman, A., Banciu, M., Vágási, C.I., 2015. Seasonal patterns and relationships among coccidian infestations, measures of oxidative physiology, and immune function in free-living house sparrows over an annual cycle. Physiol. Biochem. Zool. 88, 395–405.

Pastor, N., Baos, R., López-Lázaro, M., Jovani, R., Tella, J.L., Hajji, N., Hiraldo, F., Cortés, F., 2004. A 4 year follow-up analysis of genotoxic damage in birds of the Doñana area (south west Spain) in the wake of the 1998 mining waste spill. Mutagenesis 19, 61–65. https://doi.org/10.1093/mutage/geg035.

Rich, E.L., Romero, L.M., 2005. Exposure to chronic stress downregulates corticosterone responses to acute stressors. Am. J. Phys. Regul. Integr. Comp. Phys. 288, R1628–R1636.

Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 140, 73–79.

Romero, L.M., Dickens, M.J., Cyr, N.E., 2009. The reactive scope model - a new model integrating homeostasis, allostasis, and stress. Horm. Behav. 55, 375–389.

Romero, L.M., Platts, S.H., Schoech, S.J., Wada, H., Crespi, E., Martin, L.B., 2015. Understanding stress in the healthy animal – potential paths for progress. Stress 18, 491–497.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Schwartz, T.S., Bronikowski, A.M., 2013. Dissecting molecular stress networks: identifying nodes of divergence between life-history phenotypes. Mol. Ecol. 22, 739–756. https://doi.org/10.1111/j.1365-294X.2012.05750.x.

Sicolo, M., Tringali, M., Fumagalli, P., Santagostino, A., 2010. *Columba livia* as a sentinel species for the assessment of urban air genotoxicity. Arch. Environ. Contam. Toxicol. 59, 484–491. https://doi.org/10.1007/s00244-010-9488-3.

Sinha, A., Chu, T.T.T., Dao, M., Chandramohanadas, R., 2015. Single-cell evaluation of red blood cell bio-mechanical and nano-structural alterations upon chemically induced oxidative stress. Sci. Rep. 5, 1–8. https://doi.org/10.1038/srep09768.

Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., Gros, F., Royer, C., Massemin, S., Criscuolo, F., 2013. Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of aging. Front. Zool. 10, 33.

RStudio Team, 2015. RStudio: Integrated Development for R.

Wingfield, J.C., Vleck, C.M., Moore, M.C., 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. J. Exp. Zool. 264, 419–428.

Zhang, Z., Cheng, J., Xu, F., Chen, Y., Du, J., Yuan, M., Zhu, F., Xu, X., Yuan, S., 2011. Red blood cell extrudes nucleus and mitochondria against oxidative stress. IUBMB Life 63, 560–565. https://doi.org/10.1002/iub.490.